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The effect of genetic immunization of neonatal mice was tested with a plasmid vector expressing the rabies virus glycoprotein. Mice inoculated within 24 hr after birth with the plasmid DNA developed antibodies as well as T helper cells to the rabies virus glycoprotein. The response could not be distinguished from that seen upon vaccination of adult mice. Taken together, these data clearly show that the immune system, known to be prone to induction of immunological tolerance to some antigens applied during the early neonatal period, can readily respond to rabies virus glycoprotein induced by a plasmid vector.

INTRODUCTION

Vaccination is currently the most efficacious medical intervention for preventing human disease. Vaccines have eradicated variola major and have significantly reduced the incidence of a number of infectious childhood diseases such as rubella and poliomyelitis. Traditionally, vaccines are based on either attenuated or inactivated pathogens. Not all infectious agents can be attenuated with the high degree of reliability required of vaccines that aim at protecting healthy humans against a threat they might never encounter. Inactivated vaccines are safer, but as a rule, fail to induce cytolytic T cells that are crucial in limiting the spread of numerous viruses, facultatively intracellular bacteria, and some parasites.

Furthermore, inactivated vaccines rarely induce long-term protection. A novel vaccine approach based on immunization with plasmid vectors encoding a foreign protein under the control of a strong promoter (Tang *et al.,* 1992) might overcome some of the shortcomings of attenuated or inactivated vaccines. Vector DNA was shown to be taken up by cells upon intramuscular or intradermal inoculation (Davis *et al.,* 1993; Williams *et al.,* 1991; Wolff *et al.,* 1990), causing expression of the vector-encoded antigens, which in turn results in stimulation of an immune response (Frynan *et al.,* 1993; Ulmer *et al.,* 1993; Wang *et al.,* 1993; Xiang *et al.,* 1994).

Intriguingly, the immune response upon genetic immunization is exceptionally long-lasting in mice that upon inoculation with a single dose of vector were shown to maintain immunological memory for life (Davis *et al.,* 1993, Yakauckas *et al.,* 1993). In contrast to inactivated pathogens, proteins encoded by plasmid vectors are synthesized within transfected cells, leading to the association of antigenic fragments with major histocompatibility complex (MHC) class I determinants and hence to induction of cytolytic T cells (Ulmer *et al.,* 1993). Vector vaccines thus seem to fulfill many of the requirements expected of good vaccines. They carry none of the risks associated with live attenuated vaccines and thus far they have been shown to be safe (although further long-term studies are required to ascertain the lack of unexpected side effects). They induce a full spectrum of immune responses, including antibodies, T helper cells, and cytolytic T cells, and they provide (at least in small rodents) life-long immunity. Another wanted trait in vaccines is their ability to induce immunity in very young individuals before they come in contact with the pathogens. The immune system of neonates is partially immature; immunization with some antigens, such as alloantigens, expressed on lymphoid cells induces tolerance (Billingham *et al.,* 1953; Holan *et al.,* 1978; Streilein, 1991; Streilein and Klein, 1977); other antigens, such as polysaccharides, remain ignored due to a developmental delay in maturation of the appropriate B cell subset (Mosier *et al.,* 1977). Data presented here show that DNA vaccines given to newborn mice do not result in tolerance but rather in stimulation of an antigen-specific immune response.

MATERIAL AND METHODS

Mice

Six- to 8-week-old C3H/He mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). Groups of two females were cohoused at the Animal Facility of The Wistar Institute with one male until pregnancy was ascertained. Pups were separated from the dams at 4 weeks of age according to sex.

Cells

Baby hamster kidney (BHK)-21 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supple-

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mented with 10% fetal bovine serum (FBS), antibiotics, and glutamine. The HT-2 cell line, which proliferates to interleukin (IL)-2 and IL-4, was maintained in DMEM containing 10% FBS, 10^{-6} *M* 2-mercaptoethanol, and 10% rat concanavalin A supernatant. The CT4S cell line that responds only to IL-4 was grown in HEPES-free DMEM supplemented with 10% FBS and 2 units/ml of recombinant IL-4.

Viruses

Rabies viruses of the Evelyn Rokitniki Abelseth (ERA) strain and challenge virus standard (CVS)-11 strain were propagated on BHK-21 cells. ERA virus was purified and inactivated with β-propionolactone (BPL) as described (Xiang and Ertl, 1992). CVS-11 virus was titrated on BHK-21 cells and unpurified infectious virus was used for neutralization assays.

Plasmid

The pSG5rab.gp vector that expresses the full-length rabies virus glycoprotein under the control of the simian virus 40 promoter (Burger *et al.,* 1991) and the empty pSG5 vector were propagated in *Escherichia coli* strain DH5α in the presence of ampicillin. The plasmids were purified using the Promega Wizard Maxiprep DNA Purification system according to the manufacturer's protocol. The DNA was quantitated by agarose gel electrophoresis against a known marker.

Immunization of mice

Pups were inoculated within 24 hr after birth with 50–75 μg of the pSG5rab.gp vector or phosphate-buffered saline (PBS) or 10 μg of ERA-BPL virus given subcutaneously in a volume of 50 μl. Some of the pups were boosted at 3 months of age with 1 μg per mouse of ERA-BPL virus or with 50 μg per mouse of pSG5rab.gp vector.

Enzyme-linked immunoadsorbent assay (ELISA)

Sera obtained by retroorbital bleeding of pups at varied intervals after immunization were tested for antibodies to rabies virus on microtiter plates coated with inactivated ERA (ERA–BPL) virus, as described (Xiang and Ertl, 1992). Antibody isotypes were determined by an ELISA on ERA virus-coated plates with a 1:200 dilution of serum using the Calbiochem (LaJolla, CA) Hybridoma Subisotyping kit according to the manufacturer's specifications. We tested the reagents provided in this kit using the Hybridoma Subisotype Control kit, Mouse, also from Calbiochem. The sensitivity for detection of antibodies of different isotypes was comparable.

Neutralization assay

Virus neutralizing antibody titers were determined by neutralization of the CVS-11 strain of rabies virus prior to infection of BHK-21 cells, as described (Xiang and Ertl, 1992).

Cytokine release assay

Splenocytes (6×10^6/well) were cultured in 1.6 ml of DMEM medium supplemented with 2% FBS and 10^{-6} *M* 2-mercaptoethanol in 24-well Costar plates without antigen or with 1 μg/ml of inactivated ERA virus. Cell-free supernatants were harvested and tested for induction of proliferation of the IL-2- and -4-sensitive HT-2 cell line or the IL-4-dependent CT4S cell line, as described (Xiang *et al.,* 1994).

RESULTS

To test the effects of genetic immunization on the neonatal immune system, C3H/He mice were inoculated within 24 hr after birth with a plasmid vector expressing the rabies virus glycoprotein. Control pups were immunized either with PBS or with an equal dose of the empty pSG5 vector. The antibody response to the rabies virus antigen tested 4–6 weeks after immunization was clearly positive in most of the mice inoculated with the pSG5rab.gp vector, while pSG5-inoculated pups failed to generate titers that were significantly above those of negative control pups (results from individual mice from three separate experiments are shown in Fig. 1). Nevertheless, pSG5 vector-inoculated pups had, in most experiments, slightly elevated antibody levels, as determined by ELISA, compared to naive pups or pups inoculated with PBS, which most likely reflects the immunomodulatory activity of DNA of bacterial origin (Pisetsky, 1996).

In contrast, mice immunized as neonates with 10 μg of an inactivated rabies virus vaccine, i.e., ERA–BPL virus, failed to develop measurable antibody titers, as shown in Fig. 2.

The antibody response to genetic immunization of adult mice persists, at least in some systems, such as the one described here, for over a year. The kinetics of the antibody response of neonates to the pSG5rab.gp vector are shown in Fig. 3. Mice had good titers to rabies virus 1 month after immunization, which remained with some fluctuation at comparable levels for at least 6 months. Although in the majority of experiments, mice seroconverted after 1 month (as shown in Fig. 3), in some experiments, antibodies developed later. This might reflect variability in the inoculation method and thus a difference in the number of transfected cells.

Sera were also tested for virus neutralizing activity. Titers determined by this method were also stable for at least 5 months (the latest time point tested thus far, data not shown) ranging between virus neutralizing titers of 1:15 and 1:135 for pooled sera.

To further ensure that genetic immunization of neonates results in stimulation of an immune response, mice inoculated at birth with the pSG5rab.gp vector or the empty control plasmid were boosted at 3 months of age with a low dose of ERA–BPL virus (1 μg per mouse) given sc in 100 μl of PBS. In another experiment, mice

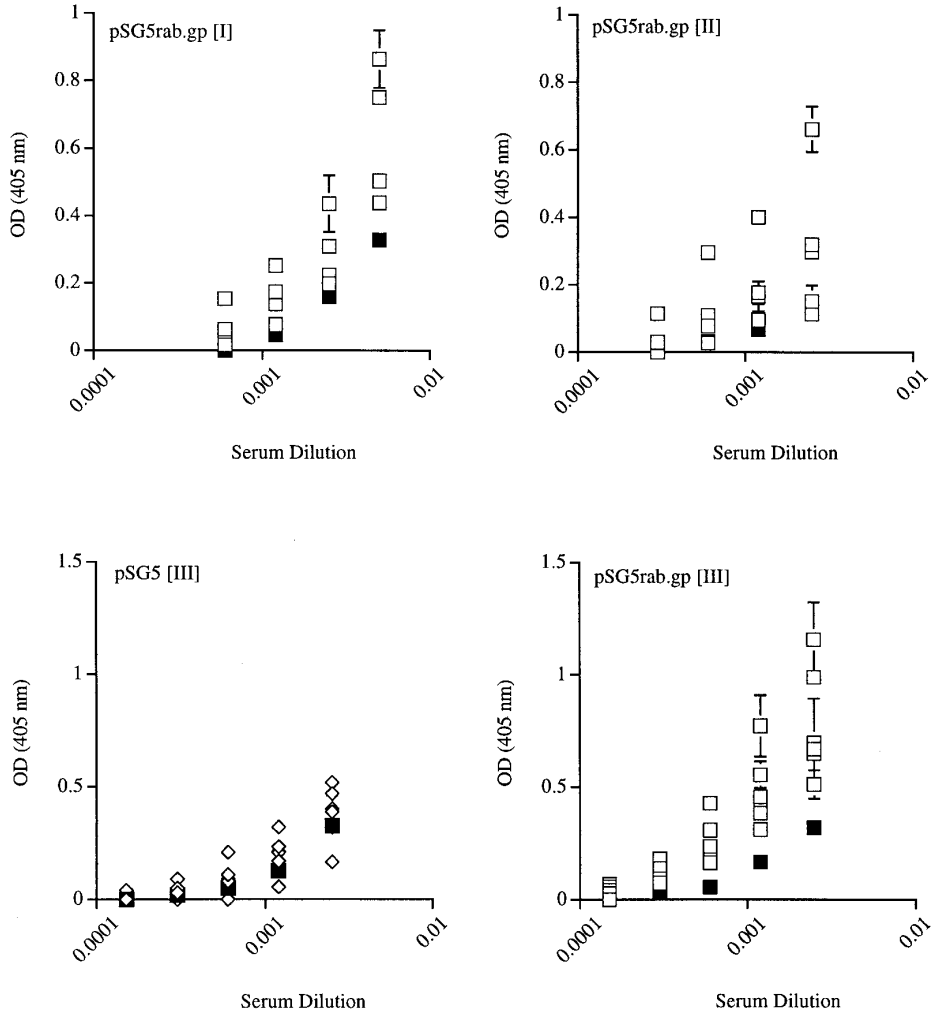

FIG. 1. The antibody response to neonatal immunization. The graph shows the results from three different experiments (I–III). Pups were immunized within 24 hr after birth with either 50–75 μg of pSG5rab.gp (□) or pSG5 (◇) vector. Control mice were inoculated with PBS. Sera of individual mice from three separate experiments shown in separate graphs were harvested 1 month after immunization and tested for antibodies to rabies virus by ELISA. Pooled serum from age-matched control pups (■) was used as a control. Data show the optical density ± standard deviations.

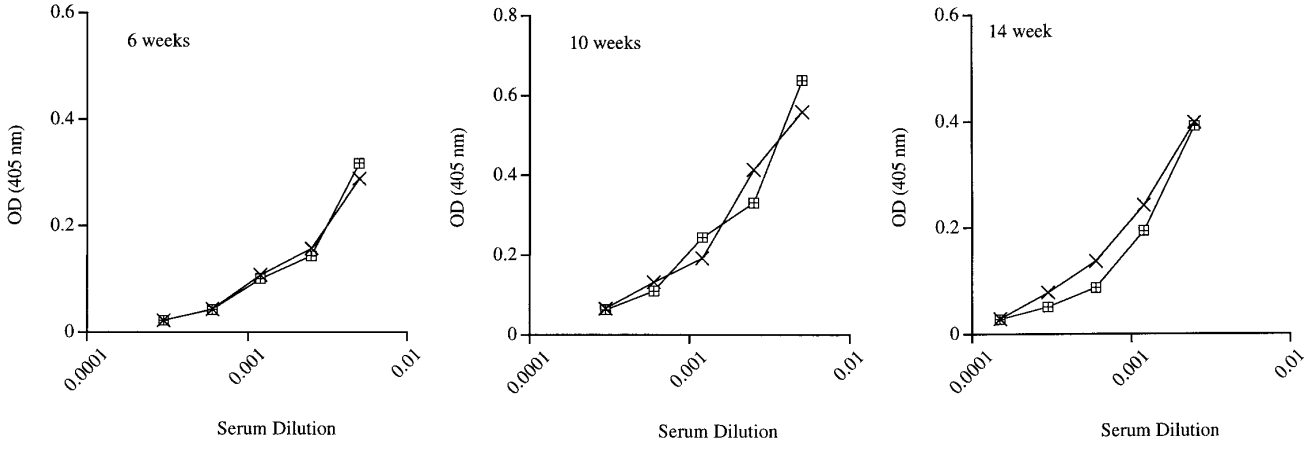

FIG. 2. The immune response of neonatal mice to inactivated rabies virus. Pups were immunized shortly after birth with 10 μg of ERA–BPL virus. Immune (⊞) as well as age-matched naive (×) pups were bled 6, 10, and 14 weeks later, and antibody titers to rabies virus were determined by an ELISA. Data were analyzed by Student *t* test (significance > 0.5).

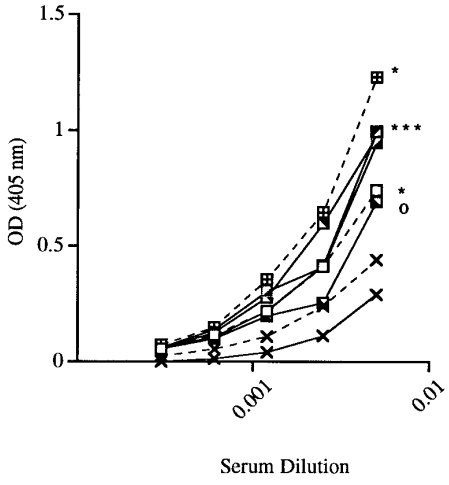

FIG. 3. The kinetics of the antibody response to neonatal immunization. Pups, immunized as described in the legend to Fig. 1, were bled 1 (◩), 2 (◥), 3 (◧), 4 (◪), 6 (⊞), and 8 (□) months later. Antibody titers were determined in two separate ELISAs (----, ———). Data were analyzed by a Student *t* test (□ ⊞ ◩ ◥ ◪ significance < 0.01; ◧ significance < 0.05).

were boosted with 50 μg of pSG5rab.gp vector given im at 2 months of age. Mice were bled 14 days later (ERA–BPL virus) or 3 months later (pSG5rab.gp) and antibody titers were determined by an ELISA. As shown in Fig. 4, pups vaccinated at birth with the pSG5rab.gp plasmid developed higher antibody titers to either vaccine used for booster immunization compared to pups vaccinated at birth with the pSG5 control vector, thus confirming that neonatal immunization of pups has a priming effect on the B cell response to rabies virus.

Induction of an immune response to rabies virus was also confirmed by partial protection of vaccinated neonates upon challenge with rabies virus; 6 of 13 mice vaccinated as neonates with the pSG5rab.gp vector survived a challenge with rabies virus given at 3–4 months of age, which caused mortality in all of the control animals (6 of 6).

One recent publications (Sarzotti *et al.,* 1996) reported that neonates, dependent on the dose of the administered antigen, respond preferentially with a Th2-like immune response characterized by lack of stimulation of cytolytic T cells. Another publication using protein antigen reported qualitatively similar responses in adult and neonatal mice (Forsthuber *et al.,* 1996). Adult mice respond to genetic immunization with a Th0/Th1-like response (Xiang *et al.,* 1992) in which T helper cells secreting IL-2 and interferon-γ and B cells producing antibodies of the IgG2a isotype predominate. To further characterize the immune response of neonates to the genetic vaccine, the isotype profile of the rabies virus-specific antibodies was determined. As shown in Fig. 5, the response was predominated by antibodies of the IgG2a isotype, with some IgG1 and IgG2b. A similar antibody isotype profile was seen upon genetic immunization of adult mice.

Pups developed a vigorous T helper cell response to rabies virus upon neonatal immunization with the pSG5rab.gp vector (Table 1). In this experiment, splenocytes of individual C3H/He mice immunized neonatally with the pSG5rab.gp vector or sham-vaccinated with PBS were tested 1 month after immunization for cytokine release in response to restimulation with inactivated rabies virus. All of the pSG5rab.gp immunized mice responded with release of cytokines comparable or superior in magnitude to that secreted by pooled lymphocytes of mice immunized 1 month earlier at 6 to 8 weeks of age with the genetic vaccine. The response tested *in vitro* initially on the IL-2- and IL-4-sensitive HT-2 cell line were shown

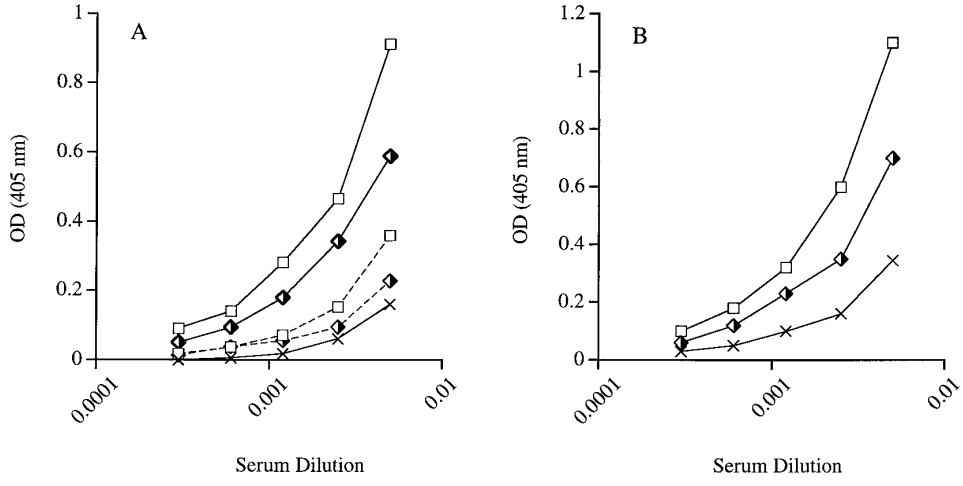

FIG. 4. Priming of the immune system by neonatal vaccination with the pSG5rab.gp vector. Mice were immunized at birth with 50 μg of pSG5rab.gp or pSG5. In one experiment (A) mice were bled at 2 months of age and boosted with 50 μg of pSG5rab.gp. Mice were bled 3 months later and antibody titers of sera before and after boost were determined [before boost: pSG5 (– – ◆ – –); pSG5rab.gp (– – □ – –); after boost: pSG5 (— ◆ —); pSG5rab.gp (—□—). In a second experiment (B), pups were vaccinated at 3 months of age with 1 g of ERA–BPL virus. They were bled 14 days later and serum antibody titers were determined [pSG5/ERA-BPL (◆); pSG5rab.gp/ERA-BPL (□). ×, normal mouse serum.

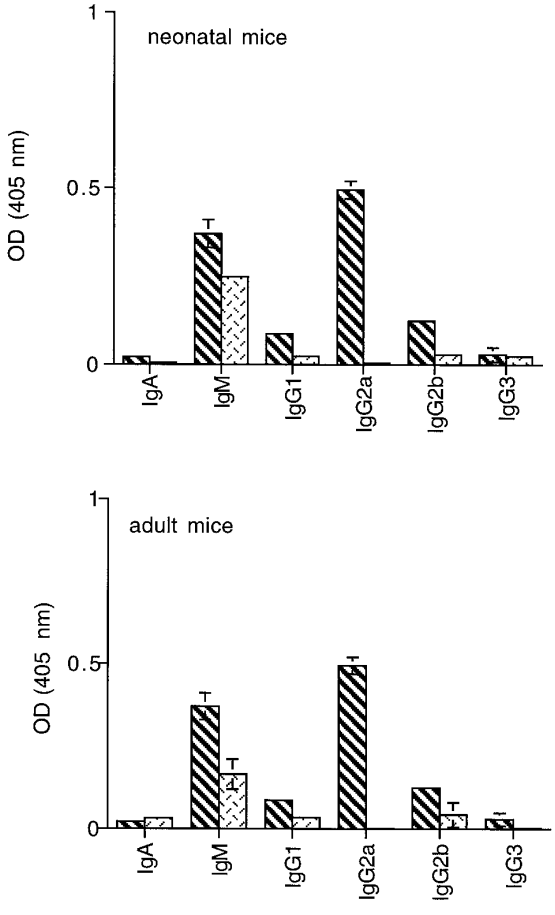

FIG. 5. The antibody isotypes induced by neonatal genetic immunization. The isotypes of antibodies to rabies virus from sera of naive mice (⊠), or from mice immunized at birth or at 6–8 weeks of age with 50 μg of pSG5rab.gp (▨) were determined by ELISA using a 1:200 dilution of serum.

to consist of IL-2; IL-4 could not be detected in the supernatants of restimulated lymphocyte cultures using either the IL-4-dependent CT4S cells as an indicator cell line or monoclonal antibodies to IL-2 and IL-4 for inhibition of activation of the HT-2 cells (method described in Xiang *et al.,* 1993). Splenocytes furthermore released low levels of interferon-γ upon *in vitro* restimulation (data not shown). This cytokine profile confirms that genetic immunization of neonates does not induce a Th2-type immune response but rather a Th0/Th1 response.

DISCUSSION

Data presented here show that a genetic vaccine expressing a viral antigen, i.e., the rabies virus G protein, induces upon inoculation of neonatal mice an anti-viral immune response consisting of T helper cells and antibodies with a predominance of antibodies of the IgG2a isotype, as seen upon genetic immunization of adult mice. Although the majority of pups vaccinated at birth with the pSG5rab.gp vaccine developed antibodies, some pups failed to respond or developed only marginal titers. By the same token, the magnitude of the T helper cell response was fairly inhomogeneous, with stimulation indices ranging from 18 to 86, which presumably reflects variability in the antigenic load expressed by transfected cells upon sc inoculation of plasmid DNA.

Neonatal inoculation of some antigen, such as those expressed on allogeneic or semiallogeneic lymphoid cells, can induce tolerance, allowing subsequent transplantation of skin grafts syngeneic to the donor lymphocytes (Billingham *et al.,* 1953). Inoculation of neonatal mice with some viruses, such as lymphocytic choriomeningitis virus, causes unresponsiveness of cytolytic T cells and concomitant stimulation of B cells (Oldstone *et al.,* 1967), resulting in viral persistence. Other antigens, such as the hepatitis B virus vaccine in human infants, induce immunity even if given during the early neonatal period. Results presented here show that neonatal exposure to antigen, as presented upon inoculation of plasmid DNA, can result in specific T and B cell responses. Induction of immunity in neonatal mice was also described in three recently published papers using a minor histocompatibility antigen, i.e., H-Y (Ridge *et al.,* 1996), a live murine retrovirus (Sarzotti *et al.,* 1996), or the protein antigen hen egg lysozyme (HEL) (Forsthuber *et al.,* 1996). Vaccination of newborn children to several infectious diseases would be highly advantageous, but first requires clarification of parameters causing activation versus tolerance of the neonatal immune system. High doses of antigen, as shown by Sarzotti *et al.* (1996), seem to favor induction of Th2 cells with apparent tolerance of cytolytic T cells.

TABLE 1

T Helper Cell Response to Neonatal Immunization

	[^{3}H]TdR incorporation (cpm ± SD)	
Immunization	Medium	ERA-BPL
pSG5rab.gp	74 ± 2	5491 ± 132
pSG5rab.gp	55 ± 6	1135 ± 97
pSG5rab.gp	51 ± 11	4389 ± 248
pSG5rab.gp	55 ± 5	991 ± 17
PBS	58 ± 17	53 ± 9
PBS	133 ± 4	138 ± 11
PBS	104 ± 2	84 ± 20
PBS	139 ± 19	80 ± 5
Control[a]	109 ± 19	1163 ± 195

Note. The data present cytokine release obtained from individual mice, with the exception of the positive control ([a]mice immunized at 6 to 8 weeks of age with the pSG5rab.gp vector), in which splenocytes pooled from three mice were tested. Data reflect triplicate sample ± SD. Mice were immunized within 24 hr after birth with pSG5rab.gp vector, Adrab.gp virus, or PBS given sc. Mice were euthanized 1 month later and splenocytes were cultured for 24 hr with medium or 1 μg/ml of ERA–BPL virus. Cell-free supernatants were tested for cytokines on the HT-2 indicator cell line.

In the H-Y system (Ridge *et al.,* 1996), tolerance by high doses of antigen could be circumvented by presentation of the male antigen on dendritic cells. The authors argue that the neonatal immune system that contains only a few T cells to a given antigen is turned off by the H-Y antigen presented by a large number of foreign splenocytes, most of which lack costimulatory signals. How does the hypothesis formulated for the H-Y system (Ridge *et al.,* 1996) apply to data presented in this paper? Inoculation of mice with a plasmid vector results in expression of the antigen on comparatively few cells close to the site of inoculation. Expression of the antigen has been demonstrated histologically on cells such as muscle cells (Wolff *et al.,* 1990), but not on dendritic cells, which is likely to reflect the insensitivity of the experimental approach. Although we have postulated that the vector DNA must cause expression of antigen on APCs to stimulate an immune response (Xiang and Ertl, 1995), most of the antigen is presumably expressed by non-APCs and should thus result in tolerance rather than activation of the neonatal immune system. Stimulation of T cells occurs mainly in lymphatic tissues such as lymph nodes: Upon genetic immunization, most of the vector-transfected non-APCs will remain stationary in the peripheral tissue, while professional APCs have the mobility to migrate upon activation to draining lymph nodes, where those that are transfected will stimulate virgin T cells, resulting in a specific immune response. The number of naive T cells that encounter the antigen first on non-APCs outside the lymph nodes is presumably small compared to those that initially meet the antigen presented by dendritic cells in lymphatic tissue, thus tipping the balance of the immune system toward stimulation rather than tolerance. In the H-Y model (Ridge *et al.,* 1996), many of the male donor splenocytes have the capacity to migrate to lymphatic tissue, where they will present the antigen to naive T cells favoring their tolerance. By the same token, the mouse retrovirus shown to cause a shift toward a Th2 response if given at high doses to neonates (Sarzotti *et al.,* 1996) multiplies in a lymphatic organ, i.e., the spleen, where it will be presented by a disproportionately higher number of non-APCs than professional APCs. We thus propose that although the dose of the antigen is a contributing factor in shifting the balance of the neonatal immune system away from stimulation of a Th1-type response toward tolerance or a switch toward a Th2-type response, the localization of the antigen, i.e., in peripheral as opposed to lymphatic tissues, is likely to be of equal importance.

A plasmid vector can cause prolonged expression of antigen (Wolff *et al.,* 1992), as was shown upon im inoculation of a vector expressing a reporter protein. Data presented here thus do not allow the finite conclusion that the neonatal immune system responds immediately to these vaccines which might have persisted until further maturation of T and/or B cells. Nevertheless, in adult mice immunized with the pSG5rab.gp vector, antibodies are not detected until about 3–4 weeks after immunization (Xiang *et al.,* 1995). In most experiments, pups inoculated at birth with vector DNA seroconverted within 1 month, suggesting that activation occurred shortly after immunization.

For practical purposes, vaccination of human neonates with a single dose of a mixture of plasmid vectors expressing relevant antigens of all viruses causing common and preventable childhood infections would be highly advantageous. Immunizations with traditional vaccines are generally delayed for several months, not only to accommodate further maturation of the immune system, but also to minimize interference of active immunization by maternally transferred antibodies. The effect of maternally transferred antibodies on neonatal immunization with genetic vaccines remains to be elucidated.

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